



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A01H 4/00, 5/10, C12N 5/00 C12N 5/04, 9/00, 15/29 C07K 13/00	A1	(11) International Publication Number: WO 94/08449 (43) International Publication Date: 28 April 1994 (28.04.94)
(21) International Application Number: PCT/US93/09816 (22) International Filing Date: 13 October 1993 (13.10.93) (30) Priority data: 07/962,481 15 October 1992 (15.10.92) US (71) Applicants: THE GENERAL HOSPITAL CORPORATION [US/US]; 55 Fruit Street, Boston, MA 02114 (US). RIJKSUNIVERSITEIT [BE/BE]; Ledeganckstraat 35, B-9000 Gent (BE). (72) Inventors: GOODMAN, Howard ; 10 The Ledges Road, Newton Center, MA 02159 (US). VAN DER STRAET-EN, Dominique ; St. Pietersnieuwstraat 70, B-9000 Gent (BE). VAN MONTAGUE, Marc ; 120, rue de Stassart, B-1050 Brussels (BE).		(74) Agent: CLARK, Paul, T.; Fish & Richardson, 225 Franklin Street, Boston, MA 02110-2804 (US). (81) Designated States: AU, CA, HU, JP, NZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: CRUCIFER ACC SYNTHASE AND USES THEREOF (57) Abstract Disclosed is substantially pure DNA encoding a crucifer ACC synthase polypeptide; a promoter functional in immature plant tissues which is capable of ethylene induction; and methods of using such promoters to express recombinant proteins or RNA and to regulate ethylene-inducible events of a plant, e.g., fruit ripening or senescence, especially during early stages of plant development.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic	KR	Republic of Korea	SD	Sudan
CG	Congo	KZ	Kazakhstan	SE	Sweden
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovak Republic
CM	Cameroon	LU	Luxembourg	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	MC	Monaco	TG	Togo
CZ	Czech Republic	MG	Madagascar	UA	Ukraine
DE	Germany	ML	Mali	US	United States of America
DK	Denmark	MN	Mongolia	UZ	Uzbekistan
ES	Spain			VN	Viet Nam
FI	Finland				

- 1 -

CRUCIFER ACC SYNTHASE AND USES THEREOFBackground of the Invention

The invention relates to recombinant plant nucleic acids and polypeptides.

The gaseous plant hormone ethylene has a wide impact on plant growth and development (Yang et al., *Ann. Rev. Plant Physiol.* 35:155, 1984). Its synthesis is induced during many stages of plant life including seed germination, leaf abscission, organ senescence, and fruit ripening. Its production also rises strongly upon exposure to various stresses including wounding, excessive temperatures, drought, flooding, and exposure to certain chemicals. Many of these effects are of significant commercial importance in agriculture.

The direct precursor of ethylene in higher plants is the three-membered-ring amino acid 1-aminocyclopropane-1-carboxylic acid. The synthesis of this three-membered-ring amino acid is catalyzed by 1-aminocyclopropane-1-carboxylate synthase (*S*-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14), commonly termed ACC synthase. This synthetic step is the rate-limiting step in the pathway to ethylene production.

Summary of the Invention

In general, the invention features substantially pure DNA (for example, genomic DNA, cDNA or synthetic DNA) encoding a crucifer (for example, *Arabidopsis*) ACC synthase polypeptide. In related aspects, the invention also features a vector, a cell (e.g., a plant cell), and a transgenic plant or seed thereof which includes such a substantially pure ACC synthase DNA. In various preferred embodiments, the cell is a prokaryotic cell, for example, *E. coli* or *Agrobacterium*, or more

- 2 -

preferably, a eukaryotic cell, for example, a transformed plant cell derived from a cell of a transgenic plant.

In a second aspect, the invention features a substantially pure DNA which includes a promoter which is
5 capable of ethylene induction and which is functional in immature plant tissues.

In preferred embodiments, the promoter is an ACC synthase promoter, for example, a crucifer ACC synthase promoter such as the ACC synthase promoter of
10 Arabodopsis. In related aspects, the invention features a transgenic plant containing a transgene which includes such an ethylene-inducible promoter which is functional in immature plant tissue; and a seed and a cell from such a transgenic plant.

15 In a third aspect, the invention features a transgenic plant containing a transgene which includes a mutant crucifer ACC synthase DNA sequence; and a seed and cell from such a transgenic plant.

In a fourth aspect, the invention features a
20 method of producing a recombinant crucifer ACC synthase polypeptide which involves: (a) providing a cell transformed with DNA encoding a crucifer ACC synthase polypeptide positioned for expression in the cell; (b) culturing the transformed cell under conditions for
25 expressing the DNA; and (c) isolating the recombinant crucifer ACC synthase polypeptide.

In a fifth aspect, the invention features a method of inhibiting an ethylene-inducible event in a plant which involves: (a) providing a transgenic plant with DNA
30 encoding a crucifer ACC synthase polypeptide positioned for expression in a cell of said plant; and (b) culturing the transgenic plant under conditions for expressing the DNA. In preferred embodiments, the events inhibited include fruit ripening, fruit maturation, senescence,
35 and/or cell development.

- 3 -

In a sixth aspect, the invention features a method of inducibly producing a compound in a cell which involves: providing a cell which includes DNA encoding the compound operably linked to an ethylene-inducible promoter which is functional in immature plant tissue; and administering ethylene to the cell to induce compound production.

In a preferred embodiment, the method may further involve isolating the compound.

10 In other preferred embodiments, the compound is a recombinant protein, a protein normally produced by the cell, or an RNA molecule; the cell is a plant cell; the cell is obtained from a transgenic plant which includes one or more cells which contain the ethylene-inducible promoter of the invention as a transgene.

In a final aspect, the invention features substantially pure crucifer ACC synthase polypeptide. Preferably, the polypeptide includes a sequence substantially identical to an amino acid sequence shown in Fig 1 (SEQ ID NOS: 1 and 2). Most preferably, the polypeptide is a crucifer ACC synthase polypeptide, for example, the *Arabidopsis* ACC synthase polypeptide.

By "crucifer" is meant any plant that is classified within the Cruciferae family as commonly described in, e.g., Gray's Manual of Botany American Book Company, N.Y., 1950; *Hortus Third: A Concise Dictionary of Plants Cultivated in the U.S. and Canada*, Macmillan, 1976; or Simmons, N.W., *Evolution of Crop Plants*, 1986. The Cruciferae include many agricultural crops, including, broccoli, cabbage, brussel sprouts, rapeseed, kale, Chinese kale, cauliflower, horseradish and *Arabidopsis*.

By "ACC synthase" is meant an ACC synthase polypeptide capable of the enzymatic conversion of AdoMet(S-adenosylmethionine) to ACC (1-aminocyclopropane-

- 4 -

1-carboxylic acid) and MTR (5-methylthioribose) as described by Yang et al., supra.

By "polypeptide" is meant any chain of amino acids, regardless of length or post-translational
5 modification (e.g., glycosylation or phosphorylation).

By "substantially identical" is meant a polypeptide or nucleic acid exhibiting at least 80%, preferably 85%, more preferably 90%, and most preferably 95% homology to a reference amino acid or nucleic acid
10 sequence.

For polypeptides, the length of comparison sequences will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids. For nucleic acids,
15 the length of comparison sequences will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides.

Homology is typically measured using sequence
20 analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software matches similar sequences by assigning degrees of homology to various
25 substitutions, deletions, substitutions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine,
30 threonine; lysine, arginine; and phenylalanine, tyrosine.

By a "substantially pure polypeptide" is meant an ACC synthase polypeptide which has been separated from components which naturally accompany it. Typically, the polypeptide is substantially pure when it is at least
35 60%, by weight, free from the proteins and naturally-

- 5 -

occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, ACC synthase polypeptide. A

- 5 substantially pure ACC synthase polypeptide may be obtained, for example, by extraction from a natural source (e.g., a plant cell); by expression of a recombinant nucleic acid encoding an ACC synthase polypeptide; or by chemically synthesizing the protein.
- 10 Purity can be measured by any appropriate method, e.g., those described in column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

- A protein is substantially free of naturally associated components when it is separated from those
- 15 contaminants which accompany it in its natural state. Thus, a protein which is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be substantially free from its naturally associated components.
- 20 Accordingly, substantially pure polypeptides include those derived from eukaryotic organisms but synthesized in *E. coli* or other prokaryotes.

- By "substantially pure DNA" is meant DNA that is free of the genes which, in the naturally-occurring
- 25 genome of the organism from which the DNA of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of
- 30 a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding
- 35 additional polypeptide sequence.

- 6 -

By "mutant form of ACC synthase polypeptide" is meant a polypeptide which includes any change (in comparison to the wild-type) in the DNA sequence. These changes can arise, e.g., spontaneously by chemical energy, e.g., X-ray, or by other forms of mutagenesis, by genetic engineering, or as a result of mating or other forms of exchange of genetic information. Mutations include, e.g., base changes, deletions, insertions, inversions, translocations, or duplications. Such mutant forms of ACC polypeptides display an inactive or attenuated ACC synthase enzymatic activity, as measured by standard ACC synthase activity assays (e.g., Yu et al., Arch. Biochem. Biophys. 198: 280, 1979; Lizada et al., Anal. Biochem. 100: 140, 1979; or Van Der Straeten et al., Eur. J. Biochem. 182:639, 1989). Preferably, such mutant ACC synthase polypeptides have (a) significantly (i.e., at least 25%) decreased activity compared to the wild-type form, or (b) have significantly (i.e., at least 25%) decreased polypeptide production (e.g., by Western blot assay) as compared to the wild-type cell.

By "transformed cell" is meant a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding (as used herein) an ACC synthase polypeptide.

By "positioned for expression" is meant that the DNA molecule is positioned adjacent to a DNA sequence which directs transcription and translation of the sequence (i.e., facilitates the production of, e.g., an ACC synthase polypeptide, a recombinant protein or a RNA molecule).

By "reporter gene" is meant a gene whose expression may be assayed; such genes include, without limitation,

- 7 -

β -glucuronidase (GUS), luciferase, chloramphenicol transacetylase (CAT), and β -galactosidase.

By "promoter" is meant minimal sequence sufficient to direct transcription. Also included in the invention 5 are those promoter elements which are sufficient to render promoter-dependent gene expression inducible by ethylene in immature plant tissue; such elements may be located in the 5' or 3' regions of the native gene.

By "immature plant tissue" is meant any tissue of 10 the plant obtained between seed imbibition and germination, and the production of flowers.

By "operably linked" is meant that a gene and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules 15 (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s).

By "plant cell" is meant any self-propagating cell bounded by a semi-permeable membrane and containing a plastid. Such a cell also requires a cell wall if further 20 propagation is desired. Plant cell, as used herein includes, without limitation, algae, cyanobacteria, seeds suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores.

By "transgene" is meant any piece of DNA which is 25 inserted by artifice into a cell, and becomes part of the genome of the organism which develops from that cell. Such a transgene may include a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic 30 organism, or may represent a gene homologous to an endogenous gene of the organism.

By "transgenic" is meant any cell which includes a DNA sequence which is inserted by artifice into a cell and becomes part of the genome of the organism which 35 develops from that cell. As used herein, the transgenic

- 8 -

organisms are generally transgenic plants and the DNA (transgene) is inserted by artifice into the nuclear or plastidic genome.

By an "ethylene inducible promotor" is meant a promoter whose level of activity is increased in response to treatment with ethylene or an equivalent compound such as propylene.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Detailed Descriptions

The drawings will first be described.

Drawings

Fig. 1 shows the nucleotide and deduced amino acid sequences of the *A. thaliana* ACC synthase gene (*ATHACC1*) (SEQ ID NO: 1) and partial cDNA (*ATHACC2*) (SEQ ID NO: 2). (A) DNA and amino acid sequences of *ATHACC1* (SEQ ID NO: 1). Exons are in upper case letters; noncoding regions are in lower case letters. The G box core element, the putative CAAT and TATA boxes, and polyadenylation signals are boxed. The region of highest similarity with other ACC synthase promoters, containing a short inverted repeat, is underlined, as well as long thymine and adenine stretches. Deduced amino acids are in the one letter code. The active site region is boxed. (B) DNA and amino acid sequences of the *ATHACC2* cDNA (SEQ ID NO: 2).

Fig. 2 shows a genomic DNA gel blot analysis of *A. thaliana*, *Oryza sativa* (Cv. Taipei), *Lycopersicon esculentum* (Cv. Orlando), and *Nicotiana tabacum*. Panel A shows from left to right: *A. thaliana* (ecotype C24), 1 μ g total DNA; *A. thaliana*, 1 μ g nuclear DNA; rice, 3 μ g nuclear DNA; tomato, 5 μ g total DNA; tobacco, 20 μ g nuclear DNA. DNA was digested with BglII (B) or EcoRI

- 9 -

(E). The filter was hybridized at 65°C with a ³²P-labeled 2.2-kb BamHI fragment of the *ATHACC1* gene. Exposure was for 12 hours on flash-sensitized film. Panel B shows from left to right: *A. thaliana* (ecotype *Lansberg erecta*) XbaI and EcoRI digest. The filter was hybridized at 60°C with a PCR fragment 3,540-3,905 base pairs, most of which covered the 3' untranslated region. Exposure was for 5 days on flash-sensitized film.

Fig. 3 is a reverse transcription-PCR analysis of the *ATHACC1* gene on total RNA from different organs of *Arabidopsis* and from mature plants after ethylene exposure. In Panel A PCR analysis was carried out using a primer specific for *ATHACC1* (PCR3 and PCR14). Lane 1, 500 base pair marker; lane 2, young leaves; lane 3, roots; lane 4, flowers; lane 5, mature green siliques; lane 6, ripe siliques; lane 7, etiolated seedlings; lane 8, mature plants; lane 9, as in 8, but after two hours of ethylene exposure; lane 10, four hours; lane 11, eight hours; lane 12, 12 hours of ethylene exposure. In Panel B PCR analysis was carried out as in Panel A but with nonspecific primers (PCR19 and PCR20). Lanes are numbered as for Panel A.

Fig. 4 is a reverse transcription-PCR analysis of expression of the *ATHACC1* gene performed on total RNA from wounded, young *Arabidopsis* leaves. In Panel A, PCR analysis was carried out using a primer specific for *ATHACC1* (PCR22 and PCR24). Lane 1, young leaves; lane 2, after 30 minutes wounding; lane 3, 60 minutes; lane 4, 90 minutes; lane 5, 2 hours; lane 6, 3 hours; lane 7, 4 hours; lane 8, 8 hours; lane 9, control on 5 ng cosmid DNA carrying *ATHACC1*. In Panel B PCR analysis was carried out as in Panel A but with nonspecific primers (PCR19 and PCR20). Lanes are numbered as for Panel A.

There now follows a description of the cloning and characterization of an *Arabidopsis* ACC synthase genomic

- 10 -

DNA and cDNA useful in the instant invention. This example is provided for the purpose of illustrating the invention, and should not be construed as limiting.

Isolation of Arabidopsis ACC Synthase Genomic DNA and

5 cDNA

A cosmid library of *A. thaliana* (ecotype Columbia) constructed by standard techniques was screened (also by standard techniques) using degenerate oligonucleotides derived from a tomato ACC synthase peptide sequence (Van
10 Der Straeten et al., supra). Isolated genomic sequences were subcloned into the pUC18 plasmid, and DNA sequence analysis was performed by the Sanger method. The library screening led to the isolation of the *AthACC1* gene and its identification by similarity to other ACC synthases.

15 Fig. 1A presents the genomic sequence of the *AthACC1* gene (5613 bp (SEQ ID NO: 1). The sequence covers 1432 bp upstream from the initiation codon and 1993 bp downstream from the stop codon. Putative CAAT and TATA boxes (Joshi, Nucl. Acids Res. 15:6643 (1987),
20 as well as the potential polyadenylation site (Dean et al., Nucleic Acids Res. 14:2229, 1986), are indicated. The gene contains three introns; consensus dinucleotides are located at their boundaries (Csank et al., Nucleic Acids Res. 18:5133, 1990). The position of the exon-
25 intron junctions are identical to those in the tomato ACC synthase genes (Rottmann et al., J. Mol. Biol. 222:937, 1991). When comparing the promoter region of *AthACC1* with the promoter of the *CPACC1A* encoding gene of zucchini (Huang et al., Proc. Natl. Acad. Sci. USA,
30 88:7021, 1991), the highest similarity was found in the region from 525 to 730. Interestingly, this region is partially overlapping with the region of highest similarity with the *Lycopersicon esculentum* *LE-ACC2* promoter (Yip et al., Proc. Natl. Acad. Sci. USA,
35 81:2475, 1992) 661-827 bp in *ATHACCI*). A 13-bp-long

- 11 -

sequence in *ATHACCI* (903-915) was also found in the fourth exon of the zucchini gene and with 77% similarity; far upstream, in the promoter of the *LE-ACC2* gene. Comparison of the *ATHACCI* promoter with other ethylene-responsive promoters (Van Der Straeten et al., *Plant Genetic Engineering*, eds., Biswas, B.B. and Harris, J.R., Plenum, NY pp. 279-326, 1991) revealed significant similarity (70-80%) in several short stretches of the 5' region. The wound-inducible promoters, *winla* (Stanford et al., *Mol. Gen. Genet.*, 215:200, 1989) and *wunla* (Logeman et al., *Mol. Gen. Genet.* 219:81, 1989), were 91% similar to *ATHACCI* in the regions 918-929 bp and 1170-1180 bp, respectively. No significant similarities with auxin-responsive elements were found. It is worth noting that a *myc*-like binding site (Blackwell et al., *Science* 230:1149, 1990) resembling the G-box core is present at position 340, found in several light-regulated promoters (Giuliano et al., *Proc. Natl. Acad. Sci. USA* 85:7089, 1988; Schulze-Lefert et al., *EMBO J.* 8:651, 1989), in the abscisic acid response element of the wheat *Em* gene (29), and in the *LE-ACC2* promoter (Rottmann et al., *supra*). In addition, both the 5'-untranslated region and intron 2 of *ATHACCI* contain long thymine (Kosambi, *Ann. Eugen.* 12:172, 1944, 36 nucleotides) and adenine stretches (15 nucleotides, close to the start codon).

As indicated in Fig. 1, the genomic clone *AthACC1*, encodes a polypeptide of 496 amino acids. This polypeptide has a predicted molecular mass of 54.6 kDa and a *Pi* of 7.3. The 12 amino acids of the active-site region are identical to those of tomato (*TACCI* or *pCVV4A*; Van Der Straeten et al., *supra*), winter squash (Nakajima et al., *Plant Cell Physiol* 31:1021, 1991), and zucchini (Sato et al., *J. Biol. Chem.* 266:3752, 1991) ACC synthases. Moreover, in all ACC synthases so far cloned, 11 of the 12 amino acids involved in the binding of the

- 12 -

substrate and the cofactor of amino transferases are conserved. This result supports the hypothesis that ACC synthases and aminotransferases are evolutionary related (Rottman et al., supra). Table I gives an overview of the amino acid and nucleotide sequence similarities of ACC synthases from various species of which full-length clones have been isolated.

TABLE I

	Enzyme	Nucleotide Identity, %	Amino Acid Identity, %	Ref.
10	TACC1 (tomato)	68	75	Van Der Straeten et al., Proc. Natl. Acad. Sci., USA, 87:4589, 1190
15	TACC2 (tomato)	66	71	Olson et al. Proc. Natl. Acad. Sci., USA, 88:5340, 1991
20	CMW33 (winter squash)	67	71	Nakajima et al., Plant Cell Physiol., 31:1021, 1990
25	CMA101 (winter squash)	68	64	Nakagawa et al., Plant Cell Physiol., 32:1153, 1991
30	CPACCIA (zucchini)	67	72	Huang et al., Proc. Natl. Acad. Sci., USA, 88:7021, 1991
	CARACC3 (carnation)	65	73	Park et al., Plant Mol. Biol. 18:388, 1992

35 A flower-specific cDNA library of ecotype C24 was constructed using standard techniques. The library was screened (also using standard techniques) with a 2.2-kilobase (kb) BamHI fragment of the *A. thaliana* ACC synthase cDNA (*AthACC1*) gene covering the first 140 amino acids of the coding region and the two first introns.

40 A partial cDNA of another *Arabidopsis* ACC synthase gene was isolated from a flower-specific cDNA library using a 2.2-kb BamHI fragment of *AthACC1* as a probe and

- 13 -

was designated *AthACC2*. Its sequence is shown in Fig. 1B (SEQ ID NO: 2). The 159 nucleotides are 79% identical to the corresponding region of *AthACC1*. This level of sequence difference cannot be readily accounted for by the difference in ecotypes (Columbia vs. C24). Deduced amino acids in this region are 83% identical.

Arabidopsis ACC Synthase Multigene Family and Its Relationship to Other Plant ACC Synthase Genes

The existence of several ACC synthase genes in *Arabidopsis* was confirmed by genomic DNA gel blots (Fig. 2). The *AthACCI* gene is a member of the ACC synthase gene family but without high similarity to any other member. Hybridization of the *AthACC1* 2.2-kb BamHI fragment to both total and nuclear *Arabidopsis* DNA under high-stringency conditions revealed a BglII fragment at 2.7 kb and two EcoRI fragments of 5.0 and 6.5 kb, each with half the intensity of the BglII band (Fig. 2A). This is a single gene pattern corresponding to the restriction map of *AthACC1*. Confirmation was obtained by hybridizing *Arabidopsis* genomic DNA with the 1.1-kb BamHI fragment (covering approximately 75% of the coding region); as well as with a 350 bp PCR generated fragment, the sequence of which resides mostly in the 3' untranslated region (Fig. 2B). In the latter cases a single band was observed at 6.5 kb in the EcoRI and at 12 kb in the XbaI lanes. However, when repeated under low-stringency conditions (53°C) with the 2.2- and 1.1-kb BamHI fragments covering the coding region as probes, several extra bands became apparent, indicating the existence of related members of the ACC synthase gene family. In addition, it could be concluded that *AthACCI* is relatively divergent from ACC synthases in rice, tomato, and tobacco, because no cross-hybridization was visible under high stringency conditions (Fig. 2A), whereas faint bands appeared under lower stringency.

- 14 -

From the aforementioned description and the nucleic acid sequence shown in Fig 1, the isolation of any crucifer cDNA encoding an ACC synthase polypeptide is easily accomplished through the use of standard

5 hybridization screening techniques (see, e.g., Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, 1989).

Restriction Fragment Length Polymorphism (RFLP) Mapping of the *AthACC1* Gene

10 RFLP mapping analysis was done by the procedure detailed by Nam et al., *Plant Cell* 1:699, 1989, using the cosmid carrying the *AthACC1* gene as a probe. Segregation data were analyzed with the MAPMAKER computer program (Lander et al., *Genomics* 1:174, 1987), and the maximum
15 likelihood recombination fractions for each pair of adjacent markers were transformed into centimorgan distances with the Kosambi function (Kosambi, *supra*).

Using the restriction enzyme *Cla*I, one Columbia polymorphic band and one Landsberg erecta polymorphic
20 band showed appropriate segregation and mapped to the same locus. This RFLP has been designated *pvv4* and maps to the telomere proximal region at the top of chromosome 1 at 0.0 centimorgans on a revised RFLP. It is 37.4 centimorgans above (telomere proximal) RFLP 5972 (which
25 mapped at 0.0) in the published map of Nam et al. (*supra*).

Study of Expression of *ATHACC1* as Determined by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

A. thaliana (ecotype C24) plants were grown at
30 22°C and 60% relative humidity under white-fluorescent light (photoperiod 16 hr light/8 hr dark, at a fluence rate of 75 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$). Young leaves from 3- to 5-week-old plants were wounded with a scalpel. Senescent leaves were from 8- to 12-week-old plants. Flower samples

- 15 -

contained only flowers with white, non-senescent petals. Mature-ripe siliques presented a yellow to light-brown coloration and had mature seeds. Mature-green siliques did not show any sign of chlorophyll loss. Flowering *A. thaliana* plants (6- to 8-week old) were either immediately frozen or, for ethylene inductions, placed in a sealed container and subjected to a continuous flow of 9 liter/hr of ethylene at 10 ppm for 2-12 hr. Auxin treatments were done by soaking 7-day old light grown seedlings in 0.5 mM indoleacetic acid (IAA) in 50 mM sodium phosphate buffer, pH 7.0, for 4 hr.

Total RNA was isolated as reported (Rodriguez-Pousada et al., Technique 2:292, 1990). RT-PCR was done as in Goblet et al., Nucleic Acids Res. 17:2144, 1989, with minor modifications. In summary, 10 µg of total RNA (accurately quantified) was mixed with the 3' oligonucleotide in 50 µl of 1X buffer (67.2 mM Tris-HCl (pH 8.8), 16.6 mM (NH₄)₂SO₄, 2 mM MgCl₂, bovine serum albumin (nuclease free) at 1.68 mg/ml, 0.72% 2-mercaptoethanol) and incubated at 85°C for 5 min, followed by 5 min at 45°C. The tubes were put on ice and 50 µl of a reaction mixture containing the 5' oligonucleotide, 1X buffer, 2.5 units of Taq polymerase (Beckman, Somerset, NJ), 16 units of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI), 20 units of RNasin (Promega), and 0.2 mM of each dNTP were added. Reverse transcription was done by incubating the tubes at 40°C for 45 min immediately followed by the PCR reaction; 30-35 cycles of 1.5 min at 94°C, 1.5 min at 50°C and 45 min at 72°C. Reactions were done in a PHC-2 thermocycler (Techne, Cambridge, U.K.).

The PCR products were analyzed by 0.8-1% agarose/TAE gel electrophoresis and blotted on Hybond-N membrane. Three pairs of oligonucleotides were used as primers:

- 16 -

Pair 1:

PCR14 5' TATAGTCTTTCTAAAGATATGGGACTT 3' (bp 2953-2979) (SEQ ID NO: 3)
 PCR3 5' GTCGTCGGAACTTAGTCGA 3' (bp 2297-2316) (SEQ ID NO.: 4);

Pair 2 (covering highly conserved regions, non-specific):

5 PCR20 5'CTCATTCCCTCCCGTACTA 3' (bp 2297-2316) (SEQ ID NO: 5)
 PCR19 5'CTCTAAAACCAAGGAGTCCC 3' (bp 2992-2973) (SEQ ID NO: 6);

Pair 3 (specific for *AthACC1*):

PCR22 5'TCGACTAAGTTCCACGAC 3' (bp 3537-3556) (SEQ ID NO: 7)
 PCR24 5 GTCGAAATTGAATTATTCCA 3' (bp 3758-3738) (SEQ ID NO: 8).

10 All data are the result of two independent experiments.

Due to the lack of sensitivity of Northern (RNA) blotting in detecting very low abundance mRNAs (as *AthACC1* mRNAs), RT-PCR was used for analysis of mRNA levels (Deidow et al., Gene Anal. Techn. 11:636, 1989; 15 Buck et al., BioTechniques 11:636, 1991). To allow quantitative comparison within each experiment, the number of PCR cycles was kept low. In certain cases Northern blots were done with a probe predicted to produce constant signals under given induction 20 conditions. Different sets of oligonucleotides derived from the coding sequences or the 3'-untranslated region of the *AthACC1* gene were synthesized to monitor levels of all ACC synthase mRNAs or *AthACC1* mRNA alone. Fig. 3 presents a DNA gel blot of a RT-PCR reaction on different 25 total RNA samples using primers PCR3 and PCR14, which most likely specifically amplify *AthACC1* cDNA because primer PCR3 resides in the 3' end of the coding region where ACC synthases are known to be highly divergent. The signal was very high in young leaves and in flowers 30 but barely visible in roots and absent in siliques and etiolated seedlings (Fig. 3A, lanes 1-7). The same pattern was found when the conserved oligonucleotides PCR19 and PCR20 were used (Fig. 3B, lanes 1-7).

When mature plants were exposed to a continuous 35 flow of ethylene at 10 ppm, an early induction could be

- 17 -

seen (2hr, Fig. 3A, lane 9) but the induction almost returned to basal level after 8 hr (lane 11). Remarkably, control plants did not show any signal (Fig. 3A, lane 8). The same pattern was observed when oligonucleotide pair 3 was used. When mature plants were exposed to a continuous flow of 10 ppm ethylene, an early induction occurred; a first peak occurred at 2 hr, and a second, strong induction occurred at 12 hr. Exposure of 7-day-old light-grown seedlings to indoleacetic acid did not lead to any significant induction of the *ATHACC1* gene.

In Fig. 4, the effect of wounding on *Arabidopsis thaliana* ACC synthase mRNA levels is shown. Fig. 4A presents a DNA gel blot of samples treated with the oligonucleotide pair 3, which specifically amplifies *AthACC1* mRNA. The signals obtained between 30-min and 8-hr wounding did not vary significantly. However, a clear induction was seen when the nonspecific set of oligonucleotides was used (pair 2); a peak occurred 4 hr after wounding (Fig. 4B).

Thus, in general, we describe the cloning of a gene encoding a member of the *A. thaliana* ACC synthase gene family (*AthACC1*) and one partial cDNA corresponding to another ACC synthase (*AthACC2*). The existence of a multigene family in *Arabidopsis* was confirmed by genomic DNA gel blots that also indicate that the *AthACC1* gene is distantly related to other family members. An analysis of the promoter sequence revealed similarities with several other promoters. Most noteworthy was the homology with the promoter of the tomato ACC synthase *LE-ACC2* gene (Rottman et al., supra) and the zucchini *CP-ACC1a* gene (Huang et al., supra), in the region 661-730 bp. In addition a G-box core element was found far upstream (340-345 bp). In both cases, their possible involvement in gene regulation remains to be provided.

- 18 -

Comparison of the predicted amino acid sequence with other ACC synthase proteins revealed the same common features presented earlier (Dong et al., Plant Cell Physiol. 32:25, 1991), and similarities ranged between 71
5 and 75% (Table I).

The exact mechanisms underlying ethylene biosynthesis by induction of ACC synthase genes is unclear. From physiological data (Yang et al., supra), it can be argued that at least three classes of ACC
10 synthases could exist -- one ripening and senescence-related, one auxin-induced, and one stress-induced. However, a recent report on ACC synthase genes in tomato has indicated that the individual genes are not assigned to only one function (Yip et al., supra). The data
15 presented here support the existence of a complex regulatory pattern of the ACC synthase gene family in *Arabidopsis*. The *AthACCI* gene was expressed prominently in young leaves and flowers but was not expressed in leaves of mature plants nor in mature green or ripe
20 siliques (Fig. 3). The *AthACC1* gene was also shut down in rosette leaves with the onset of the reproductive phase. Interestingly, *AthACC1* mRNA levels seemed to be influenced by ethylene itself. Upon ethylene exposure of mature plants, the *AthACC1* mRNA accumulated after 2 hr.,
25 whereas the ACC synthase gene family showed a biphasic activation with peaks after 2 and 12-hr treatment (Fig. 3). Although the *AthACC1* gene appeared to be switched off in senescent leaves, it remains to be determined whether it has any role in the onset of senescence or in
30 the early senescence phase remains to be determined. In addition, *AthACC1* was shown not to be wound-inducible, at least not in young tissue. This contrasted with the pattern of accumulation observed for the ACC synthase mRNAs in general, where a peak was detected after 4 hr
35 (Fig. 4).

- 19 -

ACC Synthase Polypeptide Expression

Polypeptides according to the invention may be produced by transformation of a suitable host cell with all or part of an ACC synthase cDNA (e.g., the cDNA described above) in a suitable expression vehicle.

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems may be used to provide the recombinant protein. The precise host cell used is not critical to the invention. The ACC synthase polypeptide may be produced in a prokaryotic host, e.g., *E. coli*, or in a eukaryotic host, e.g., *Saccharomyces cerevisiae*, mammalian cells (e.g., COS 1 or NIH 3T3 cells), or any of a number of plant cells including, without limitation, algae, tree species, ornamental species, temperate fruit species, tropical fruit species, vegetable species, legume species, monocots, dicots, or in any plant of commercial or agricultural significance. Particular examples of suitable plant hosts include Chlamydomonas, Conifers, Petunia, Tomato, Potato, Tobacco, *Arabidopsis*, Lettuce, Sunflower, Oilseed rape, Flax, Cotton, Sugarbeet, Celery, Soybean, Alfalfa, *Medicago*, Lotus, Vigna, Cucumber, Carrot, Eggplant, Cauliflower, Horseradish, Morning Glory, Poplar, Walnut, Apple, Asparagus, Rice, Corn, Millet, Onion, Barley, Orchard grass, Oat, Rye, and Wheat.

Such cells are available from a wide range of sources including: the American Type Culture Collection (Rockland, MD); Chlamydomonas Culture Collection, (Duke University), Durham, North Carolina; or from any of a number seed companies, e.g., W. Atlee Burpee Seed Co. (Warminster, PA), Park Seed Co. (Greenwood, SC), Johnny Seed Co. (Albion, ME), or Northrup King Seeds (Harstville, SC). Descriptions and sources of useful host cells are also found in Vasil I.K., *Cell Culture and*

- 20 -

Somatic Cell Genetics of Plants, Vol I, II, III
Laboratory Procedures and Their Applications Academic
Press, New York, 1984; Dixon, R.A., *Plant Cell Culture-A
Practical Approach*, IRL Press, Oxford University, 1985;
5 Green et al., *Plant Tissue and Cell Culture*, Academic
Press, New York, 1987; Gasser and Fraley, *Science*
244:1293, 1989.

For prokaryotic expression, DNA encoding an ACC
synthase of the invention is carried on a vector operably
10 linked to control signals capable of effecting expression
in the prokaryotic host. If desired, the coding sequence
may contain, at its 5' end, a sequence encoding any of
the known signal sequences capable of effecting secretion
of the expressed protein into the periplasmic space of
15 the host cell, thereby facilitating recovery of the
protein and subsequent purification. Prokaryotes most
frequently used are various strains of *E. coli*; however,
other microbial strains may also be used. Plasmid
vectors are used which contain replication origins,
20 selectable markers, and control sequences derived from a
species compatible with the microbial host. Examples of
such vectors may be found in Pouwels et al. (*supra*) or
Ausubel et al. (*supra*). Commonly used prokaryotic
control sequences (also referred to as "regulatory
25 elements") are defined herein to include promoters for
transcription initiation, optionally with an operator,
along with ribosome binding site sequences. Promoters
commonly used to direct protein expression include the
beta-lactamase (penicillinase), the lactose (lac) (Chang
30 et al., *Nature* 198: 1056, 1977), the tryptophan (Trp)
(Goeddel et al., *Nucl. Acids Res.* 8: 4057, 1980) and the
tac promoter systems as well as the lambda-derived P_L
promoter and N-gene ribosome binding site (Simatake et
al., *Nature* 292:128, 1981).

- 21 -

For eukaryotic expression, the method of transformation or transfection and the choice of vehicle for expression of the ACC synthase will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al. (supra); Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989; Gelvin et al., *Plant Molecular Biology Manual*, Kluwer Academic Publishers, 1990; Kindle, K., *Proc. Natl. Acad. Sci., USA* 87:1228, 1990; Potrykus, I., *Annu. Rev. Plant Physiol. Plant Mol. Biology* 42:205, 1991; and BioRad (Hercules, CA) Technical Bulletin #1687 (Biolistic Particle Delivery Systems). Expression vehicles may be chosen from those provided, e.g., in *Cloning Vectors: A Laboratory Manual* (P.H. Pouwels et al., 1985, Supp. 1987); Gasser and Fraley (supra); Clontech Molecular Biology Catalog (Catalog 1992/93 Tools for the Molecular Biologist, Palo Alto, CA); and the references cited above.

One preferred eukaryotic expression system is the mouse 3T3 fibroblast host cell transfected with a pMAMneo expression vector (Clontech, Palo Alto, CA). pMAMneo provides: an RSV-LTR enhancer linked to a dexamethasone-inducible MMTV-LTR promotor, an SV40 origin of replication which allows replication in mammalian systems, a selectable neomycin gene, and SV40 splicing and polyadenylation sites. DNA encoding an ACC synthase polypeptide would be inserted into the pMAMneo vector in an orientation designed to allow expression. The recombinant ACC synthase protein would be isolated as described below. Other preferable host cells which may be used in conjunction with the pMAMneo expression vehicle include COS cells and CHO cells (ATCC Accession Nos. CRL 1650 and CCL 61, respectively).

Alternatively, an ACC synthase polypeptide is produced by a stably-transfected mammalian cell line.

- 22 -

A number of vectors suitable for stable transfection of mammalian cells are available to the public, e.g., see Pouwels et al. (supra); methods for constructing such cell lines are also publicly available, e.g., in Ausubel et al. (supra). In one example, cDNA encoding the ACC synthase polypeptide is cloned into an expression vector which includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, the ACC synthase-encoding gene into the host cell chromosome is selected for by inclusion of 0.01-300 μ M methotrexate in the cell culture medium (as described in Ausubel et al., supra). This dominant selection can be accomplished in most cell types. Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene. Methods for selecting cell lines bearing gene amplifications are described in Ausubel et al. (supra); such methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. DHFR-containing expression vectors commonly used for this purpose include pCVSEII-DHFR and pAdd26SV(A) (described in Ausubel et al., supra). Any of the host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR⁻ cells, ATCC Accession No. CRL 9096) are among the host cells preferred for DHFR selection of a stably-transfected cell line or DHFR-mediated gene amplification.

Most preferably, an ACC synthase polypeptide is produced by a stably-transfected plant cell line or by a transgenic plant. A number of vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants are available to the public; such vectors are described in Pouwels et al. (supra), Weissbach and Weissbach (supra), and Gelvin et al. (supra). Methods for constructing such cell lines are described in, e.g., Weissbach and Weissbach (supra),

- 23 -

and Gelvin et al. (*supra*). Typically, plant expression vectors include (1) a cloned plant gene under the transcriptional control of 5' and 3' regulatory sequences and (2) a dominant selectable marker. Such plant expression vectors may also contain, if desired, a promoter regulatory region (e.g., one conferring inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

An example of a useful plant promoter according to the invention is a caulimovirus promoter, e.g., a cauliflower mosaic virus (CaMV) promoter. These promoters confer high levels of expression in most plant tissues, and the activity of these promoters is not dependent on virally encoded proteins. CaMV is a source for both the 35S and 19S promoters. In most tissues of transgenic plants, the CaMV 35S promoter is a strong promoter (see, e.g., Odell et al., *Nature* 313: 810, 1985). The CaMV promoter is also highly active in monocots (see, e.g., Dekeyser et al., *Plant Cell* 2:591, 1990; Terada and Shimamoto, *Mol. Gen. Genet.* 220:389, 1990). Moreover, activity of this promoter can be further increased (i.e., between 2-10 fold) by duplication of the CaMV 35S promoter (see e.g., Kay et al., *Science* 236:1299, 1987; Ow et al., *Proc. Natl. Acad. Sci., USA* 84: 4870, 1987; and Fang et al., *Plant Cell* 1: 141, 1989).

Other useful plant promoters include, without limitation, the nopaline synthase promoter (An et al., *Plant Physiol.* 88: 547, 1988) and the octopine synthase promoter (Fromm et al., *Plant Cell* 1: 977, 1989).

- 24 -

For certain applications, it may be desirable to produce the ACC synthase gene product in an appropriate tissue, at an appropriate level, or at an appropriate developmental time. Thus, there are an assortment of

5 gene promoters, each with its own distinct characteristics embodied in its regulatory sequences, shown to be regulated in response to the environment, hormones, and/or developmental cues. These include gene promoters that are responsible for (1) heat-regulated

10 gene expression (see, e.g., Callis et al., Plant Physiol. 88: 965, 1988), (2) light-regulated gene expression (e.g., the pea *rbcS*-3A described by Kuhlemeier et al., Plant Cell 1: 471, 1989; the maize *rbcS* promoter described by Schaffner and Sheen, Plant Cell 3: 997,

15 1991; or the chlorophyll a/b-binding protein gene found in pea described by Simpson et al., EMBO J. 4: 2723, 1985), (3) hormone-regulated gene expression (e.g., the abscisic acid responsive sequences from the *Em* gene of wheat described Marcotte et al., Plant Cell 1:969, 1989),

20 (4) wound-induced gene expression (e.g., of *wun1* described by Siebertz et al., Plant Cell 1: 961, 1989), or (5) organ-specific gene expression (e.g., of the tuber-specific storage protein gene described by Roshal et al., EMBO J. 6:1155, 1987; the 23-kDa zein gene from

25 maize described by Schernthaner et al., EMBO J. 7: 1249, 1988; or the French bean β -phaseolin gene described by Bustos et al., Plant Cell 1:839, 1989).

Plant expression vectors may also optionally include RNA processing signals, e.g, introns, which have

30 been shown to be important for efficient RNA synthesis and accumulation (Callis et al., Genes and Dev. 1: 1183, 1987). The location of the RNA splice sequences can dramatically influence the level of transgene expression in plants. In view of this fact, an intron may be

35 positioned upstream or downstream of an ACC synthase

- 25 -

polypeptide-encoding sequence in the transgene to modulate levels of gene expression.

In addition to the aforementioned 5' regulatory control sequences, the expression vectors may also
5 include regulatory control regions which are generally present in the 3' regions of plant genes (Thornburg et al., Proc. Natl Acad. Sci USA 84: 744, 1987; An et al., Plant Cell 1: 115, 1989). For example, the 3' terminator region may be included in the expression vector to
10 increase stability of the mRNA. One such terminator region may be derived from the PI-II terminator region of potato. In addition, other commonly used terminators are derived from the octopine or nopaline synthase signals.

The plant expression vector also typically
15 contains a dominant selectable marker gene used to identify those cells that have become transformed. Useful selectable genes for plant systems include genes encoding antibiotic resistance genes, for example, those encoding resistance to hygromycin, kanamycin, bleomycin,
20 G418, streptomycin or spectinomycin. Genes required for photosynthesis may also be used as selectable markers in photosynthetic-deficient strains. Finally, genes encoding herbicide resistance may be used as selectable markers; useful herbicide resistance genes include the
25 bar gene encoding the enzyme phosphinothricin acetyltransferase and conferring resistance to the broad spectrum herbicide Basta® (Hoechst AG, Frankfurt, Germany).

Efficient use of selectable markers is facilitated
30 by a determination of the susceptibility of a plant cell to a particular selectable agent and a determination of the concentration of this agent which effectively kills most, if not all, of the transformed cells. Some useful concentrations of antibiotics for tobacco transformation
35 include, e.g., 75-100 µg/ml (kanamycin), 20-50 µg/ml

- 26 -

(hygromycin), or 5-10 $\mu\text{g/ml}$ (bl omycin). A useful strat gy for selection of transformants for herbicide resistance is described, e.g., by Vasil et al., *supra*.

It should be readily apparent to one skilled in 5 the art of molecular biology, especially in the field of plant molecular biology, that the level of gene expression is dependent, not only on the combination of promoters, RNA processing signals and terminator elements, but also on how these elements are used to 10 increase the levels of selectable marker gene expression.

Plant Transformation

Upon construction of the plant expression vector, several standard methods are accessible for introduction of the recombinant genetic material into the host plant 15 for the generation of a transgenic plant. These methods include (1) *Agrobacterium*-mediated transformation (*A. tumefaciens* or *A. rhizogenes*) (see, e.g., Lichtenstein and Fuller In: *Genetic Engineering*, vol 6, PWJ Rigby, ed, London, Academic Press, 1987; and Lichtenstein, C.P., and 20 Draper, J., In: *DNA Cloning*, Vol II, D.M. Glover, ed, Oxford, IRI Press, 1985), (2) the particle delivery system (see, e.g., Gordon-Kamm et al., *Plant Cell* 2:603, 1990; or BioRad Technical Bulletin 1687, *supra*), (3) microinjection protocols (see, e.g., Green et al., 25 *supra*), (4) polyethylene glycol (PEG) procedures (see, e.g., Draper et al., *Plant Cell Physiol* 23:451, 1982; or e.g., Zhang and WU, *Theor Appl Genet* 76:835, 1988), (5) liposome-mediated DNA uptake (see, e.g., Freeman et al., *Plant Cell Physiol* 25: 1353, 1984), (6) electroporation 30 protocols (see, e.g., Gelvin et al *supra*; Dekeyser et al. *supra*; or Fromm et al *Nature* 319: 791, 1986), and (7) the vortexing method (see, e.g., Kindle *supra*).

The following is an example outlining an *Agrobacterium*-mediated plant transformation. The general 35 process for manipulating genes to be transferred into the

- 27 -

genome of plant cells is carried out in two phases. First, all the cloning and DNA modification steps are done in *E. coli*, and the plasmid containing the gene construct of interest is transferred by conjugation into 5 *Agrobacterium*. Second, the resulting *Agrobacterium* strain is used to transform plant cells. Thus, for the generalized plant expression vector, the plasmid contains an origin of replication that allows it to replicate in *Agrobacterium* and a high copy number origin of 10 replication functional in *E. coli*. This permits facile production and testing of transgenes in *E. coli* prior to transfer to *Agrobacterium* for subsequent introduction into plants. Resistance genes can be carried on the vector, one for selection in bacteria, e.g., 15 streptomycin, and the other that will express in plants, e.g., a gene encoding for kanamycin resistance or an herbicide resistance gene. Also present are restriction endonuclease sites for the addition of one or more transgenes operably linked to appropriate regulatory 20 sequences and directional T-DNA border sequences which, when recognized by the transfer functions of *Agrobacterium*, delimit the region that will be transferred to the plant.

In another example, plants cells may be 25 transformed by shooting into the cell tungsten microprojectiles on which cloned DNA is precipitated. In the Biolistic Apparatus (Bio-Rad, Hercules, CA) used for the shooting, a gunpowder charge (22 caliber Power Piston Tool Charge) or an air-driven blast drives a plastic 30 macroprojectile through a gun barrel. An aliquot of a suspension of tungsten particles on which DNA has been precipitated is placed on the front of the plastic macroprojectile. The latter is fired at an acrylic stopping plate that has a hole through it that is too 35 small for the macroprojectile to go through. As a

- 28 -

result, the plastic macroprojectile smashes against the stopping plate and the tungsten microprojectiles continue toward their target through the hole in the plate. For the instant invention the target can be any plant cell, tissue, seed, or embryo. The DNA introduced into the cell on the microprojectiles becomes integrated into either the nucleus or the chloroplast.

Transfer and expression of transgenes in plant cells is now routine practice to those skilled in the art. It has become a major tool to carry out gene expression studies and to attempt to obtain improved plant varieties of agricultural or commercial interest.

Transgenic Plant Regeneration

Plants cells transformed with a plant expression vector can be regenerated, e.g., from single cells, callus tissue or leaf discs according to standard plant tissue culture techniques. It is well known in the art that various cells, tissues and organs from almost any plant can be successfully cultured to regenerate an entire plant; such techniques are described, e.g., in Vasil supra; Green et al., supra; Weissbach and Weisbacch, supra; and Gelvin et al., supra.

In one particular example, a cloned ACC synthase polypeptide under the control of the 35S CaMV promoter and the nopaline synthase terminator and carrying a selectable marker (e.g., kanamycin resistance) is transformed into *Agrobacterium*. Transformation of leaf discs (e.g., of tobacco leaf discs), with vector-containing *Agrobacterium* is carried out as described by Horsch et al. (Science 227: 1229, 1985). Putative transformants are selected after a few weeks (e.g., 3 to 5 weeks) on plant tissue culture media containing kanamycin (e.g. 100 μ g/ml). Kanamycin-resistant shoots are then placed on plant tissue culture media without

- 29 -

hormones for root initiation. Kanamycin-resistant plants are then selected for greenhouse growth. If desired, seeds from self-fertilized transgenic plants can then be sowed in a soil-less media and grown in a greenhouse.

- 5 Kanamycin-resistant progeny are selected by sowing surfaced sterilized seeds on hormone-free kanamycin-containing media. Analysis for the integration of the transgene is accomplished by standard techniques (see, e.g., Ausubel et al. supra; Gelvin et al. supra).
- 10 Transgenic plants expressing the selectable marker are then screened for transmission of the transgene DNA by standard immunoblot and DNA detection techniques. Each positive transgenic plant and its transgenic progeny are unique in comparison to other transgenic plants
- 15 established with the same transgene. Integration of the transgene DNA into the plant genomic DNA is in most cases random and the site of integration can profoundly effect the levels, and the tissue and developmental patterns of transgene expression. Consequently, a number of
- 20 transgenic lines are usually screened for each transgene to identify and select plants with the most appropriate expression profiles.

Transgenic lines are evaluated on levels of transgene expression. Expression at the RNA level is

25 determined initially to identify and quantitate expression-positive plants. Standard techniques for RNA analysis are employed and include PCR amplification assays using oligonucleotide primers designed to amplify only transgene RNA templates and solution hybridization

30 assays using transgene-specific probes (see, e.g., Ausubel et al., supra). The RNA-positive plants are then analyzed for protein expression by Western immunoblot analysis using ACC synthase specific antibodies (see, e.g., Ausubel et al., supra). In addition, *in situ*

35 hybridization and immunocytochemistry according to

- 30 -

standard protocols can be done using transgene-specific nucleotide probes and antibodies, respectively, to localize sites of expression within transgenic tissue.

Once the recombinant ACC synthase polypeptide is expressed in any cell or in a transgenic plant (e.g., as described above), it may be isolated, e.g., using affinity chromatography. In one example, an anti-ACC synthase antibody (e.g., produced as described in Ausubel et al., supra, or by any standard technique) may be attached to a column and used to isolate the polypeptide. Lysis and fractionation of ACC synthase-producing cells prior to affinity chromatography may be performed by standard methods (see, e.g., Ausubel et al., supra). Once isolated, the recombinant protein can, if desired, be further purified, e.g., by high performance liquid chromatography (see, e.g., Fisher, *Laboratory Techniques In Biochemistry And Molecular Biology*, eds., Work and Burdon, Elsevier, 1980).

These general techniques of polypeptide expression and purification can also be used to produce and isolate useful ACC fragments or analogs.

In other applications, however, expression of the transgene in the plant cell or the transgenic plant may be the desired result. These include applications such as ACC synthase-controlled regulation of fruit ripening or altering the normal development of the plant.

ACC Synthase Promoters

Since plant gene expression varies in accordance with developmental stages of different cell types and in response to different environmental factors and hormonal cues, the promoters (including the gene regulatory sequences) of the present invention are most useful for applications aimed at improving or engineering plant varieties of agricultural or commercial interest. As

- 31 -

described above, crucifer ACC synthase expression is developmentally regulated during the early stages of development. It is induced by ethylene and is not responsive to auxin or wounding. Accordingly, the ACC synthase nucleic acid sequences of the present invention include regulatory sequences that promote expression of a gene in response to the phytohormone, ethylene, at specific developmental times.

To identify the minimal sequences sufficient to promote ethylene-inducible expression in immature plant tissues, functional analyses of the ACC synthase regulatory sequences are performed. These assays may be carried out using transiently transformed plant cells or transgenic plants according to standard methods (see, e.g., Gelvin et al., supra). Likely candidates for regulatory elements include those sequences shown to be important for expression of other eukaryotic genes, e.g., upstream activating sequences or enhancer elements (which may control, e.g., tissue specific or inducible expression; Wasyllyk B. CRC Rev 23, 77, 1988). Other regulatory elements useful for gene expression may include RNA processing signals, 3' terminator regions, and the gene-encoding sequence itself.

To identify particular regulatory elements of the ACC synthase promoter, 5' deletion fragments of the promoter region are constructed and analyzed in either transient assays or in vivo in transgenic plants. Construction of chimeric transgenes carrying 5' deletion fragments are produced by standard methods (see, e.g., Ausubel et al, supra). The wild-type promoter and deletion fragments are then fused to a reporter gene, for example, the β -glucuronidase gene (GUS) (see, e.g., Jefferson, Plant. Mol. Biol. Rep. 316: 387, 1987) in a plant expression vector and introduced into a host by any established method (as described above). These expression

- 32 -

v ctors are then transformed into *Agrobacterium* followed by transformation of the plant material, e.g., leaf discs (see, e.g., Gelvin et al. supra). Regenerated shoots are selected on medium containing, e.g., kanamycin. After
5 rooting, transgenic plantlets are transferred to soil and grown in a growth room.

Primary transformants are then assayed for ethylene-induced GUS activity at early stages of development either by quantitating GUS activity or by
10 histochemical staining as described below. Untransformed plants are taken as controls. Ethylene inductions can be carried out by placing the transformant in a sealed container and subjecting it to a continuous flow of approximately 1-50 L/hr or 1-100ppm ethylene for 1-24
15 hrs.

Fluorometric analysis of GUS activity can be performed in any plant cell protoplast or transgenic plant according to standard methodologies. Alternatively, preparations of crude plant extracts can
20 be assayed as described, e.g., by Jefferson (supra), using extracts standardized for protein concentration (see, e.g., Bradford, Anal. Biochem. 72: 248, 1976). GUS levels in different plant tissues are assayed by enzymatic conversion of 4-methylumbelliferyl glucuronide
25 to 4-methylumbelliferone, which is quantified with a fluorimeter (e.g., Perkin-Elmer LS 2B, Norwalk, CT). Typically, the fluorimeter is set at 455 nm emission and 365 nm excitation wavelengths. GUS activity is generally expressed as picomoles per milligram of protein per
30 minute (see, e.g., Jefferson supra).

Alternatively, GUS activity can be assayed by in situ histochemical staining, e.g., as follows. Whole tissues and thin sections from transgenic plants and untransformed control plant tissue can be stained by
35 incubation with 5-bromo-4-chloro-3-indoyl B-D-glucuronic

- 33 -

acid (X-gluc; Research Organics, Inc., Cleveland OH) as described by Jefferson et al (EMBO J 6: 3901, 1987) and Gallagher (GUS Protocols, 1992). Tissue sections are incubated at 37°C in 2 mM X-gluc in 0.1 M NaPO₄ (pH 7.0),
5 and then sectioned. GUS activity in a transformed plant is easily identified by the presence of an indigo blue precipitate within the cells expressing the reporter gene. Stained material is optionally examined microscopically using bright-field and dark-field optics.

10 Use

Introduction of crucifer ACC synthase into a transformed plant cell facilitates the manipulation of developmental events controlled by ethylene. For example, transgenic plants of the instant invention
15 expressing crucifer ACC synthase might be used to alter, simply and inexpensively, ethylene biosynthesis and thereby inhibit or regulate, e.g., senescence, or fruit ripening, or any number of other plant developmental events induced by ethylene.

20 The instant invention also provides nucleic acid regulatory sequence elements capable of being induced by ethylene at early stages of plant development. Such sequences are useful, e.g., in transgenic plants to effect expression of a variety of recombinant proteins or
25 RNA molecules. This expression may be controlled by ethylene induction and limited to specified periods of development.

Other Embodiments

The invention also includes any biologically
30 active fragment or analog of a crucifer ACC synthase polypeptide. By "biologically active" is meant possessing any in vivo or in vitro activity which is

- 34 -

characteristic of the ACC synthase polypeptide shown in Fig. 1. Because ACC synthase exhibits a range of physiological properties and because such properties may be attributable to different portions of the ACC synthase molecule, a useful ACC synthase fragment or ACC synthase analog is one which exhibits a biological activity in any biological assay for ACC synthase activity, for example, those assays described by Yang et al., supra. In particular, a biologically active ACC synthase fragment or analog possesses at least 10%, preferably 40%, and more preferably 90% of an activity of the wild-type ACC synthase polypeptide.

Preferred analogs include ACC synthase polypeptides (or biologically active fragments thereof) whose sequences differ from the wild-type sequence only by conservative amino acid substitutions, for example, substitution of one amino acid for another with similar characteristics (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative amino acid substitutions, deletions, or insertions which do not abolish the polypeptide's biological activity.

Analogous can differ from naturally occurring ACC synthase polypeptide in amino acid sequence or can be modified in ways that do not involve sequence, or both. Analogous of the invention will generally exhibit at least 70%, preferably 80%, more preferably 90%, and most preferably 95% or even 99%, homology with a segment of 20 amino acid residues, preferably 40 amino acid residues, or more preferably the entire sequence of a naturally occurring ACC synthase polypeptide sequence.

Alterations in primary sequence include genetic variants, both natural and induced. Also included are analogs that include residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., β or

- 35 -

γ amino acids. Alternatively, increased stability may be conferred by cyclizing the peptide molecule. Also included in the invention are crucifer ACC synthase polypeptides modified by *in vivo* or *in vitro* chemical derivatization of polypeptides, including acetylation, methylation, phosphorylation, carboxylation, or glycosylation.

In addition to substantially full-length polypeptides, the invention also includes biologically active fragments of the polypeptides. As used herein, the term "fragment", as applied to a polypeptide, will ordinarily be at least 20 residues, more typically at least 40 residues, and preferably at least 60 residues in length. Fragments of ACC synthase polypeptide can be generated by methods known to those skilled in the art. The ability of a candidate fragment to exhibit a biological activity of ACC synthase can be assessed by those methods described herein. Also included in the invention are ACC synthase polypeptides containing residues that are not required for biological activity of the peptide, e.g., those added by alternative mRNA splicing or alternative protein processing events.

- 36 -

SEQUENCE LISTIN**(1) GENERAL INFORMATION:**

- (i) APPLICANT: Van Der Stra t n, Dominique et al.
- (ii) TITLE OF INVENTION: CRUCIFER ACC SYNTHASE AND USES THEREOF .
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: Fish & Richardson
(B) STREET: 225 Franklin Street
(C) CITY: Boston
(D) STATE: Massachusetts
(E) COUNTRY: U.S.A.
(F) ZIP: 02110-2804
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
(B) COMPUTER: IBM PS/2 Model 502 or 55SX
(C) OPERATING SYSTEM: MS-DOS (Version 5.0)
(D) SOFTWARE: WordPerfect (Version 5.1)
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER: 07/962,481
(B) FILING DATE: October 15, 1992
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER:
(B) FILING DATE:
- (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Paul T.Clark
(B) REGISTRATION NUMBER: 30,162
(C) REFERENCE/DOCKET NUMBER: 00786/161001
- (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: (617) 542-5070
(B) TELEFAX: (617) 542-8906
(C) TELEX: 200154

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:**(i) SEQUENCE CHARACTERISTICS:**

- 37 -

(A) LENGTH:	5613
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```

GATCCCAAAA AACAAATGGGT TCGTTGGTTT TGTGTGAAAG TGTAGATGTA ACGACTCCAA      60
ATAGCCAAAA GTTGATATCT TCAGTCTATA TTATTGAGAT CTTATGTCCC CCCTGTAATT      120
AATTTTCCA GCAATGCGTG CTTAAGAAT ATTGTTGAG ACTTTAGTTA TTGTAATACG      180
ACATTAGTAT AAGTAGAGCC AAAATCAGAT TTTAATATCT TTAGTCTTAA GACATGAAAC      240
AAGATTAAGA AAATACCTTG TTTTCAAAGA AACCGTTAT AAAAAGGAGG ATTTGAGTTT      300
TTGACATTCA GACGATAAAA ATTATGAACT AGGTCTAGTC ACGTGGTCGA CGCGTGAGAG      360
TTTCCGGCGT GAACTGCAAG TAAAATCAG TAGAGCATGT GATTGACTTG ACCAAAGAGT      420
CCAAACCCAC CAACTACAAA AAAAAATCAA GATATAAATA ACTAACTCTC ACTAGTCACT      480
AATATAATTT TTCATTACAA TTCATATATG ATCTACTAAC AATTGTTGTG GTTATACAAA      540
CAAAAAATTT ATTTTTCGTA AGACGGAAAT TTTGAAATCA AATTCCTCAA CACTCAAATG      600
AATATTCAGT AGTAGTTTAT CAATGACTAG ATTAGATATT TCTTAACGCC AGTCAAATTT      660
TGGAATTATG TGGAGGACGT ACGTTTATAC ATGTGCAGAC TACAACATAC CAAATGTTTT      720
ATTAAACCAA ATTACAATGT TGCAAAATGG TCTATTCTTT TGAATAATCT GATACATTTT      780
ATCTCATAAC TTTCTTCCTT TTTATTTGAA TTCAATCAA TAATATTCTC CACATCCCCA      840
ACCTTCTTTT TTTTTTTGCA TGACTAAGTA GTTTAAGGTC AACATTTTTC ATAAGAAGTT      900
GCTTAGAAAT AGCCTTGGGT TCAAATAAAA TACACATGAT TTTCCCGTTT CCACCAATAA      960
ATCCCAATGG ATTTTAATAC TGAAACGGAA ATCAATGCGA AACTATTGGA GTAAGACCAA      1020
TTTATTCATC TTAATCTACC AAATTCGATA CGATATGTTT AATACAAGGG AGATTGATGC      1080
TAGCAAAACA CAACCATCTT AATTTTTTTT TTTTTTTTTT AATTAGAAAT TCCCTTCCCA      1140
AATGGTAATT CAATCGTAAC AAAAGTACGT TTTGAAATAT TGTTTTGGAT GGAGATTTTT      1200
TCCTTGTTTC GCTTGTTACT TTTCACTTGT TTCATCAAAT CCTAACTCCT TTTATTTTGG      1260
ACCCACATC AACTTTATTT GGTCTCCTCA AGGTTTCTGT TTCAACTCCT ATATAAAGC      1320
AAATAACTCA TACGTTAATT AGTACACACC AAAAAACTT GTATAAGATC AATATCGATA      1380
CCCCCAAAA AAAAAAAAAA CAGCTACAAA GAAGTGAGAA TTGACACAGC AAATGGGTCT      1440
TCCGGGAAAA AATAAAGGTG CAGTTTTGTC GAAGATAGCG ACTAACAATC AACACGGAGA      1500
GAACTCAGAG TACTTTGATG GATGGAAAGC TTACGACAAA GATCCTTTTC ATCTTTCCCG      1560
TAACCCCAT GGGATCATCC AAATGGGTCT TGCAGAGAAT CAGGTACAGA TTATATATAA      1620
TCCAATAAAT CATGTTATAT GTTGTGTCG TTGTGCATGA ACTTCCATCT ATTAGCTATT      1680

```

- 38 -

ATATATGAAC ACGTATACAC ATCAAGCTAA TACCTTTTTT TCTTCTTTTC AAGTCAAGTT	1740
AAATACTTAA TAACACATTT TTCTAAACTT CTTACAGCTT TGCTTAGATT TGATCAAAGA	1800
TTGGGTCAAA GAGAACCCAG AAGCTTCTAT TTGCACCCTT GAAGGTATTC ATCAGTTTAG	1860
CGACATCGCT AATTTCCAAG ACTACCATGG TCTTAAGAAG TTTAGACAGG TACTATAAAT	1920
CATTCATTAT TCAGATATCT TGTAATCAGC TACGGACATA TTAGAAAAAC AATTTTTTACA	1980
TGGAAAGTTA ATAACACCTC TAAACAATCA GTTGATATGA TCTGCATAAG AAAAACAAAT	2040
TCAGTCGTGG TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTGTTA AAGATTCGTG	2100
TTGCATTTAA TTAGTAACTT ATTTTATAAA CTTATCCCTA ATATAAATTT TGAATTGAA	2160
GGCAATTGCA CATTCATGG GAAAAGCTAG AGGTGGAAGA GTGACTTTTG ATCCGGAGAG	2220
GGTGGTTATG AGCGGAGGAG CCACCGGAGC CAATGAAACA ATCATGTTCT GCCTTGCGGA	2280
TCCCGGCGAC GTTTTCCTCA TTCCCTCCCC GTACTATGCC GCGTAAGCAT TGTTAAAAAC	2340
ATTAATCACA TTTTAAAGAG AAAATAGTAC TAGTATATGA TAATGGATAA TGTTAGGAC	2400
AGATTCATT AATGTTACTT TCACATACTT TTTTGGGGTT AACAAATTCT AAATCGAAAT	2460
GAGTTATTAG TATCAAGTTT TGACTCTTTT GCCAACTTT ATCACACGTG TACGATATAT	2520
CCATTCAATA GCGGTTTTAA TTGAACGACA AGCTCTCATA CGTGTGATAA TTAATGATTT	2580
AATCCTTTCC GCAGATTGTA TAGAGACTTG AGGTGGCGGA CAGGTGTCGA GATAATCCCG	2640
GTTCTTGTT CAAGCTCCGA CAATTTCAAA TTAACCGTTG ACGCCGCGGA ATGGGCTTAT	2700
AAAAAAGCCC AAGAGTCCAA TAAAAAGTC AAAGGTCTGA TTTTGACCAA CCCATCAAAT	2760
CCACTCGGTA CAATGTTGGA TAAGGACACA CTCACGAAT TGGTCCGGTT TGTCACGAGG	2820
AAGAACATTC ACCTAGTCGT CGACGAGATC TACGCCGCCA CAGTCTTCGC CGGAGGAGAT	2880
TTCGTGAGCG TTGCTGAGGT GGTCAATGAT GTGGACATCT CCGAAGTCAA CGTTGACTTG	2940
ATTCACATTG TCTATAGTCT TTCTAAAGAT ATGGGACTTC CTGGTTTTAG AGTCGGGATA	3000
GTCTATTCTT TCAATGACTC GGTCTGTCT TCGCAAGAA AAATGTCAAG TTTCGGACTT	3060
GTTTCGTCTC AGACACAACT CATGCTTGCT TCGATGTTGT CCGATGATCA GTTTGTGGAT	3120
AATTTTCTAA TGGAAAGCTC GAGAAGGTTG GGGATAAGGC ATAAAGTTT TACCACGGGG	3180
ATCAAGAAAG CAGATATTGC TTGTTTGACA AGCAACGCTG GTTTATTTGC GTGGATGGAT	3240
TTGAGACATC TACTGAGAGA TCGTAACTCG TTTGAATCTG AGATCGAGCT TTGGCATATA	3300
ATCATCGATA GAGTTAAGCT CAATGTGTCT CCTGGCTCTT CCTTCCGTTG CACGGAACCT	3360
GGATGGTTTA GGATTTGCTT TGCCAACATG GACGATGATA CTCTCCATGT GGCCTTGGA	3420
CGGATCCAAG ATTTCTGTGC TAAGAACAAG AACAAGATCG TCGAGAAAGC ATCTGAAAAT	3480
GATCAGGTAA TCCAGAACAA GAGTGCTAAA AAGCTGAAAT GGACGCAGAC CAATCTTCGA	3540
CTAAGTTTCC GACGACTTTA CGAGGATGGT CTCTCGTCTC CAGGGATAAT GTCACCACAC	3600

- 39 -

TCACCTCTTC TCCGAGCATG AAAATCTTAA GGCATAACGT CTGAGAGATT GGATTAACCTC	3660
GTCCGCGTTT CACTCCGTGT TAATTAATCT TAAATTAGTA AGTGATTAAG TAAATGTTTT	3720
TTCTTTTCATT GTAAGATTGG AATAATTCAA TTTGACATT AGGGTTGTTT TTGACGGCCA	3780
GCTTTTTTCC TGGGGTCAAA TGGTAACTTT TAAGATTTTA TGTGTTTGAT TCTGTTTCTT	3840
TTTTCCGCTT AGGATTTTAA TCGATGGATT GTCCTAGTGG TGCTGGTGTG TAGCATATAT	3900
GCTTTTCTTA TATGTTTTTG TGTGTAATAA ATGAAACATT GTCTTTTGAT AAGGATCACC	3960
AGAGTTTATT AGTTGGGGAG GTTGATAATG TTTTGTGAGT AATGGAGGAT TTGTTAACCT	4020
AATTTATTCG ATTTTTTCTA GAACCGCATT TTCTTGTTG CCCAATACGT CACACGAGCA	4080
TGCCAACATG CCTATCCTTT TTCTAAATA ATCATTATAT GACTAAATT GAACATCCGA	4140
TTATACAGAG ATATAATCAA TAAATGCATG TTAAGTTTTA TATCTTGGA TTTGCCTTAG	4200
CCTATCATAT TGTGGGTGAT GAAAGATTCA TCAGCATTTT AGCTGCACCA AATATGATTA	4260
AATTCAACTT ATTATTTTGT TACAAAGTGA CAAATTTGCT TAGAATAATC AGCTATCAAA	4320
CATGATGACG TCTCCATCAA TTATTCAATA ATCGTCAGCT TTCTTTCCCC TTTTCTTTG	4380
TTAATGATAA ACCGTCAGCT TGAATGTTAT AGTATTTATT TTTGGTCCTC TTTTGGTAAA	4440
CCATTAGCTA TTATTTGATA AAATTTACAG ATCTCAGATT GATAAATTTG TTACATATAT	4500
TATATGCTAC TACGACTTTG TTAGGTAATT AAGTGCTGAT GGTAAGGCGT GCTTTGGGCC	4560
GCCTCTTAGC TGATATTGAT ACTACACACA CGAACAAAAA TATATATATA TAAGACAAAA	4620
ATCAAATTTG ATACTTGGA ACAACGGTTT GATCCTTTT ACACAGAATA ATATGTATCT	4680
GGATAATATA TAGATATCTC TGTCTAATTA TAATCATCGA CATTATCGTC GTCATCATCA	4740
GTCACAAGTC ACAACCAATT CATGATCATC AACAGTAGGT TACGAAACAT GATCAAGTTA	4800
TATATTTATT TTGTTTGGTA GAAAAATGA CATGGGCAAG TTTTTTTTTA TATATATATA	4860
GTGAATCCTC TTTTAAATAT TCAAGGGAAC TTTTCTGT CTTGGATTTT GTTTTGACTC	4920
TAAATAATTC AATACGGCAT AAATTGAAAA TGATGAAATA CCAAATTAAG TTTTCACATG	4980
CTCCTTTTAG GTGGCTACCT ACCAAAATGT TTTTGACATT TGCATTTGGT TTGAGCCACA	5040
ACTTGATCTA TGACATTTAC AATGCACTTG GTTACGTGAA GACTATTTTT AGTAATATAT	5100
CTTTTAACAA AAAAAAGAT ATATTTTCAA TAATCTTTTG GTGTCGAAAA AGAAGAAGT	5160
TTGTATGTGG CCGAGATACG GGCATTTTTA TTCTTAAGTG GTTTCTAGAT TTTTATTTT	5220
TTTTGTTTAG AATAAATTA GAACCTTCACT TTATGCTATT ATCCCCTGAA ATACAGGTAC	5280
ATTTGTGAAG AAATAAATA AAAATTAGAA CAATTAATAA CGCTTTACCT TCTCCTCTTA	5340
CAAAATTTCT AAAAGTGAAT CATCAGATGA TCATGAAGGC CATGCCCTTT GCTTCGCGCA	5400
AAACAGAAGT AAAGATAAAT TATCAAGTTT ACAGCTGAAA TGTTAATAAG CCGCCCAACA	5460
ACATTTATTC ACCTAAGCTA GCACCCACAT ACATTTAAAA ATATATATAT TGACCAGATT	5520

- 40 -

ATGAAAAAAC TTTGACAATA ACATAGTTAT GAAATATACA TAACCTTAAG AAGAAGATGA 5580
 CCAGGTTATG AAATAGCAAA ATCGAATAAA AAC 5613

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 496
 (B) TYPE: amino acid
 (C) STRANDEDNESS: N/A
 (D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Gly Leu Pro Gly Lys Asn Lys Gly Ala Val Leu Ser Lys Ile Ala
 1 5 10 15
 Thr Asn Asn Gln His Gly Glu Asn Ser Glu Tyr Phe Asp Gly Trp Lys
 20 25 30
 Ala Tyr Asp Lys Asp Pro Phe His Leu Ser Arg Asn Pro His Gly Ile
 35 40 45
 Ile Gln Met Gly Leu Ala Glu Asn Gln Leu Cys Leu Asp Leu Ile Lys
 50 55 60
 Asp Trp Val Lys Glu Asn Pro Glu Ala Ser Ile Cys Thr Leu Glu Gly
 65 70 75 80
 Ile His Gln Phe Ser Asp Ile Ala Asn Phe Gln Asp Tyr His Gly Leu
 85 90 95
 Lys Lys Phe Arg Gln Ala Ile Ala His Phe Met Gly Lys Ala Arg Gly
 100 105 110
 Gly Arg Val Thr Phe Asp Pro Glu Arg Val Val Met Ser Gly Gly Ala
 115 120 125
 Thr Gly Ala Asn Glu Thr Ile Met Phe Cys Leu Ala Asp Pro Gly Asp
 130 135 140
 Val Phe Leu Ile Pro Ser Pro Tyr Try Ala Ala Phe Asp Arg Asp Leu
 145 150 155 160
 Arg Trp Arg Thr Gly Val Glu Ile Ile Pro Val Pro Cys Ser Ser Ser
 165 170 175
 Asp Asn Phe Lys Leu Thr Val Asp Ala Ala Glu Trp Ala Tyr Lys Lys
 180 185 190
 Ala Gln Glu Ser Asn Lys Lys Val Lys Gly Leu Ile Leu Thr Asn Pro
 195 200 205
 Ser Asn Pro Leu Gly Thr Met Leu Asp Lys Asp Thr Leu Thr Asn Leu
 210 215 220
 Val Arg Phe Val Thr Arg Lys Asn Ile His Leu Val Val Asp Glu Ile
 225 230 235 240
 Tyr Ala Ala Thr Val Phe Ala Gly Gly Asp Phe Val Ser Val Ala Glu
 245 250 255

- 41 -

Val Val Asn Asp Val Asp Ile Ser Glu Val Asn Val Asp Leu Ile His
 260 265 270

Il Val Tyr Ser Leu Ser Lys Asp Met Gly Leu Pro Gly Phe Arg Val
 275 280 285

Gly Ile Val Tyr Ser Phe Asn Asp Ser Val Val Ser Cys Ala Arg Lys
 290 295 300

Met Ser Ser Phe Gly Leu Val Ser Ser Gln Thr Gln Leu Met Leu Ala
 305 310 315 320

Ser Met Leu Ser Asp Asp Gln Phe Val Asp Asn Phe Leu Met Glu Ser
 325 330 335

Ser Arg Arg Leu Gly Ile Arg His Lys Val Phe Thr Thr Gly Ile Lys
 340 345 350

Lys Ala Asp Ile Ala Cys Leu Thr Ser Asn Ala Gly Leu Phe Ala Trp
 355 360 365

Met Asp Leu Arg His Leu Leu Arg Asp Arg Asn Ser Phe Glu Ser Glu
 370 375 380

Ile Glu Leu Trp His Ile Ile Ile Asp Arg Val Lys Leu Asn Val Ser
 385 390 395 400

Pro Gly Ser Ser Phe Arg Cys Thr Glu Pro Gly Trp Phe Arg Ile Cys
 405 410 415

Phe Ala Asn Met Asp Asp Asp Thr Leu His Val Ala Leu Gly Arg Ile
 420 425 430

Gln Asp Phe Val Ser Lys Asn Lys Asn Lys Ile Val Glu Lys Ala Ser
 435 440 445

Glu Asn Asp Gln Val Ile Gln Asn Lys Ser Ala Lys Lys Leu Lys Trp
 450 455 460

Thr Gln Thr Asn Leu Arg Leu Ser Phe Arg Arg Leu Tyr Glu Asp Gly
 465 470 475 480

Leu Ser Ser Pro Gly Ile Met Ser Pro His Ser Pro Leu Leu Arg Ala
 485 490 495

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	159
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CAGGCGATTG CGACGTTTAT GGAGAGAGCG AGAGGCGGGC GGGTGAGGTT TGAGGCGGAG 60

AGGGTGGTGA TGAGCGGAGG AGCCACCGGA GCAAATGAGA CGATCATGTT CTGTCTTGCT 120

- 42 -

GATCCCGGCG ACGCTTTTCT CGTCCCTACT CCTTATTAT

159

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	53
(B) TYPE:	amino acid
(C) STRANDEDNESS:	N/A
(D) TOPOLOGY:	N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Gln	Ala	Ile	Ala	Thr	Phe	Met	Glu	Arg	Ala	Arg	Gly	Gly	Arg	Val	Arg
1				5					10					15	
Phe	Glu	Ala	Glu	Arg	Val	Val	Met	Ser	Gly	Gly	Ala	Thr	Gly	Ala	Asn
			20					25					30		
Glu	Thr	Ile	Met	Phe	Cys	Leu	Ala	Asp	Pro	Gly	Asp	Ala	Phe	Leu	Val
			35				40					45			
Pro	Thr	Pro	Tyr	Tyr											
			50												

What is claimed is:

- 43 -

Claims

1. Substantially pure DNA encoding a crucifer ACC synthase polypeptide.
2. The DNA of claim 1, wherein said DNA is
5 genomic DNA or said DNA is cDNA.
3. The DNA of claim 1, wherein said DNA is of the genus Arabidopsis.
4. A vector comprising the DNA of claim 1, said vector being capable of directing expression of the
10 protein encoded by said DNA in a vector-containing cell.
5. A cell which contains the DNA of claim 1 or the vector of claim 4.
6. The cell of claim 5, said cell being a plant cell.
- 15 7. A transgenic plant which contains the DNA of claim 1 or claim 4.
8. A seed from a transgenic plant of claim 7.
9. A cell from a transgenic plant of claim 7.
10. Substantially pure DNA comprising a promoter
20 capable of ethylene induction and functional in immature plant tissues.
11. The promoter of claim 10, said promoter being the ACC synthase promoter.

- 44 -

12. The promoter of claim 11, said promoter being the crucifer ACC synthase promoter.

13. The promoter of claim 12, said promoter being of the genus Arabidopsis.

5 14. A transgenic plant containing a transgene comprising a DNA sequence under the transcriptional control of the promoter of claim 10.

15. A seed from a transgenic plant of claim 14.

16. A cell from a transgenic plant of claim 14.

10 17. A transgenic plant containing a transgene comprising a mutant ACC synthase DNA sequence.

18. A seed from a transgenic plant of claim 17.

19. A cell from a transgenic plant of claim 17.

20. A method of producing a recombinant crucifer
15 ACC synthase polypeptide comprising:

(a) providing a cell transformed with DNA encoding a crucifer ACC synthase polypeptide positioned for expression in said cell;

(b) culturing said transformed cell under
20 conditions for expressing said DNA; and

(c) isolating said recombinant crucifer ACC synthase polypeptide.

21. A method of inhibiting an ethylene-inducible event in a plant comprising:

- 45 -

providing a transgenic plant with DNA encoding a crucifer ACC synthase polypeptide positioned for expression in a cell of said plant; and

culturing said transgenic plant under conditions
5 for expressing said DNA.

22. The method of claim 21, wherein said ethylene-inducible events comprise fruit ripening, fruit maturation, or senescence.

23. A method of inducibly producing a compound in
10 a cell comprising:

providing a cell which comprises DNA encoding said compound operably linked to a promoter of claim 10; and administering ethylene to said cell to induce compound production.

15 24. The method of claim 23, wherein said compound is a recombinant protein, a protein normally produced by said cell, or an RNA molecule.

25. The method of claim 23, wherein said cell is a plant cell.

20 26. The method of claim 23, wherein said cell is obtained from a transgenic plant which includes the promoter of claim 10 as a transgene.

27. A substantially pure crucifer ACC synthase polypeptide.

25 28. The polypeptide of claim 27, wherein said crucifer is of the genus Arabidopsis.

- 46 -

29. The polypeptide of claim 28, comprising an amino acid sequence substantially identical to an amino acid sequence shown in Fig. 1 (SEQ ID NOS: 1 and 2).

[illegible]

B 1 CAGGCGATTCCGACGTTTATGGAGAGCGAGGCGGGCGGGTGAGGTTTGAGCGGAGAGGGTGATGACCGGAGGAGCCACCGGAGCAAAATGAGACGATCATGTCTGCTTGTCT
Q A I A T F M E R A R G . G R V R F E A E R V V M S G G A T G A N E T I M F C L A
121 GATCCCGGCGACGCTTTTCTGTCCTACTCCTATTAT
D P G D A F L V P T P Y Y

FIG. 1

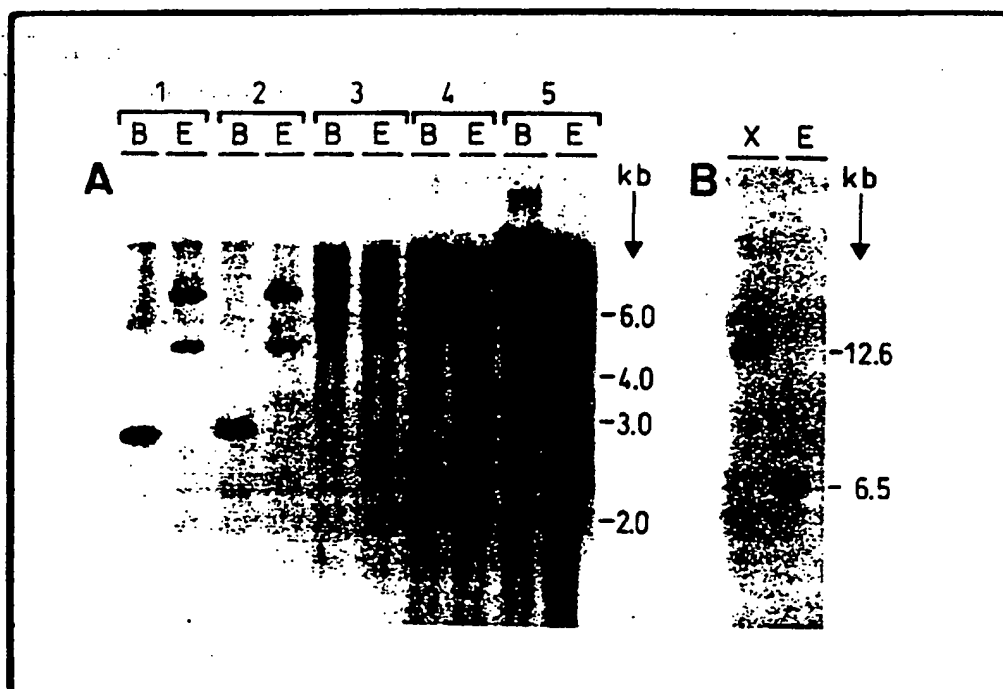


FIG. 2



FIG. 3

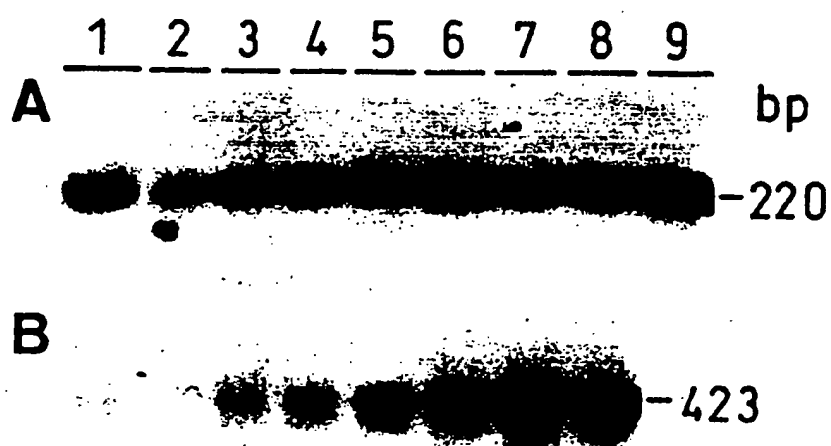


FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/09816

A. CLASSIFICATION OF SUBJECT MATTER

IPC(S) :A01H 4/00, 5/10; C12N 5/00, 5/04, 9/00, 15/29; C07K 13/00

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.6; 435/69.1, 172.3, 240.1, 240.4, 320.1; 800/205, 250; 530/370; 800/DIG 15

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature, vol. 346, Issued 19 July 1991, Hamilton et al., "Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants", pages 284-287, see the entire document.	1-9, 20-22, 27-29
Y	Science, vol. 254, Issued 18 October 1991, Oeller et al., "Reversible inhibition of tomato fruit senescence by antisense RNA", pages 437-439, see the entire document.	1-9, 20-22, 27-29

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"A" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 21 DECEMBER 1993	Date of mailing of the international search report 31 JAN 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer <i>Jill Warden for</i> CHE SWYDEN/CHERESKIN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/09816

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X P,Y	Proceedings National Academy of Science, USA, volume 89, Issued October 1992, Van der Straeten et al., "Cloning, genetic mapping, and expression analysis of an Arabidopsis thaliana gene that encodes 1-aminocyclopropane-1-carboxylate synthase", pages 9969-9973, see the entire document.	<u>1-3,5,27-29</u> 4,6-9,20-22
Y	Plant Molecular Biology, volume 18, number 4 Issued February 1992, Botella et al., "Identification and characterization of three putative genes for 1-aminocyclopropane-1-carboxylate synthase from etiolated mung bean hypocotyl segments", pages 793-797, see the entire document.	1-9,20-22, 27-29
Y	Proceedings National Academy, USA, volume 88, Issued June 1991, Olson et al., "Differential expression of two genes for 1-aminocyclopropane-1-carboxylate synthase in tomato fruits", pages 5340-5344, see the entire document.	1-9, 20-22, 27-29
Y	Journal of Molecular Biology, volume 222, Issued 20 December 1991, Rottmann et al., "1-Aminocyclopropane-1-carboxylate synthase in tomato is encoded by a multigene family whose transcription is induced during fruit and floral senescence", pages 937-961, see the entire document.	1-9, 20-22, 27-29
Y	Proceedings National Academy of Science, USA, volume 88, Issued August 1991, "Two genes encoding 1-aminocyclopropane-1-carboxylate synthase in zucchini (Cucurbita pepo) are clustered and similar but differentially regulated", pages 7021-7025, see the entire document.	1-9, 20-22, 27-29

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

536/23.6; 435/69.1, 172.3, 240.1, 240.4, 320.1; 800/205, 250; 530/370

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

Sequence search by STIC; Chemical Abstracts Online (File Biosis 1963-1993); USPTO Automated Patent System (File USPAT 1971-1993).

Search terms: ACC SYNTHASE, Crucifer, Brassica, Arabidopsis, Sequence from Figure 1.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKINGThis ISA found multiple inventions as follows:**Reasons for Holding Lack of Unity of Invention**

Group I, claims 1-9, 20-22 and 27-29 drawn to ACC synthase structural gene, vectors, transgenic plants, seeds and cells, method for producing recombinant ACC synthase protein. ACC synthase protein and method for inhibition of an ethylene inducible event.

Group II, claims 10-16 and 23-26, drawn to a promoter, transgenic plants, seeds and cells which contain the promoter, and a method of inducibly producing a compound in a cell.

Group III, claims 17-19, drawn to transgenic plants, cells and seeds containing a mutant ACC synthase.

Groups I and II contain independent and wholly separate products. The two products are unrelated structurally. Furthermore, the structural gene of Group I can be used separately from the promoter of Group II and the promoter can be used separately from the structural gene of Group I.

Groups II and III contain independent and wholly separate products. The two products are unrelated structurally. Furthermore, the promoter in the transgenic plants, cells and seeds of Group II would confer different properties on the plants, cells and seeds of Group II than the plants, cells and seeds of Group III which contain a mutant ACC synthase.

Group I and Group III are independent and distinct inventions. Group I contains products that are unrelated structurally to the products of Group III. Furthermore, the transgenic plants, cells and seeds of Group III which contain the mutated ACC synthase would be expected to have different properties than the transgenic plants, cells and seeds of Group I which contain an unmutated ACC synthase.

These inventions are distinct for the reasons given above and have acquired a separate status in the art because of their recognized divergent subject matter and are not so linked by a special technical feature within meaning of PCT Rule 13.2 so as to form a single inventive concept.

Che Swyden Chereskin, Ph.D.
Patent Examiner, Art Unit 1804